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SPECIFIC DELIVERY OF MITOMYCIN C TO THE LIVER, SPLEEN AND LUNG: NANO- AND MICROSPHERICAL CARRIERS OF GELATIN

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SUMMARY

Gelatin spherical carriers of different sizes, nanospheres (NS) with a mean diameter of 280 nm and microspheres (MS) with a mean diameter of 14.9 μm, were prepared and their potential as delivery systems for anticancer agents was evaluated.

Carriers were labeled with ^{131}I and their biodistribution showed a selective distribution of [^{131}I]NS to the liver and spleen, and [^{131}I]MS to the lung. Incorporation of mitomycin C (MMC) as a free form followed the distribution pattern of the carriers alone, but as a dextran conjugate (MMCD) induced a sustained localization; with a slight decrease of the antitumor activity.

Thus, this new formulation provided a very specific delivery system of MMC to the reticuloendothelial system and the lung with sustained localization.

INTRODUCTION

The ideal dosage form in cancer chemotherapy is the one that provides a specific delivery of anticancer agent to the tumor site in a sufficient amount, for a long period of time with no interaction with the normal tissue. Considerable efforts have been directed in our laboratory towards the development of a timed-release device which could be implanted in the closest possible proximity of a malignant tissue (Kojima et al., 1978; Hashida et al., 1978) or a carrier system which could deliver anticancer agents selectively into malignant cells thriving far from the injection site (Hashida et al., 1977a, b and 1979; Tanigawa et al., 1980).

Recently, several types of particulate carriers intended to modify the systemic distribution of soluble agents have been proposed as possible drug delivery systems. The in vivo distribution of actinomycin D or other agents has been modified by entrapping them into

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liposomes (Gregoliadis, 1974; Tanaka et al., 1975; Kimelberg et al., 1976) showing increased pharmacological activities (Mayhew et al., 1976; Kosloski et al., 1978). Albumin microspheres (Kramer, 1974; Sugibayashi et al., 1977) and polyalkylcyanoacrylate nanoparticles (Couvreur et al., 1980) have also been developed as a biodegradable particulate carrier.

The present report describes the preparation and characterization of new types of particulate carriers, gelatin nanospheres (NS) and microspheres (MS). The effect of particle size on their in vivo distribution and their potential for delivering mitomycin C (MMC) is examined. Incorporation of soluble drugs into particulate carrier together with the use of a modified form (higher molecular weight) of the free drug is a novel approach as drug carriers. The effectiveness of MMC–dextran conjugate (MMCD), a high molecular weight pro-drug of MMC (Kojima et al., 1980), entrapped in gelatin spheres is demonstrated.

MATERIALS AND METHODS

Materials

MMC was supplied from Kyowa Hakko, Japan. [^{131}I]-Labeled human serum albumin (HSA) was purchased from Daiichi Radioisotopes, Japan, with a specific activity of 0.1 mCi/mg. Alkaline-processed gelatin with an isoelectric point of about 5.1 was obtained from Nakarai Chemicals, Japan, and dextran was purchased from Pharmacia Fine Chemicals, Sweden (Dextran T-70). Non-ionic surfactants, a polyoxyethylene derivative of hydrogenated castor oil (HCO-60) and sorbitan sesquioleate (SO-15) were supplied from Nikko Chemicals, Japan. All other chemicals were of reagent grade quality and obtained commercially from Nakarai Chemicals, Japan. MMCD was synthesized as previously described (Kojima et al., 1980). One gram of the conjugate was estimated to contain 134 mg of MMC.

Animals used were male Wistar albino rats (200–220 g) and male hybrid BDF_1 mice (C57BL/6 X DBA/2; 20–23 g). P388 leukemia was supplied by Shionogi Pharmaceuticals, Japan.

Preparation of NS and MS

Gelatin spherical carriers employed in the present investigation were prepared by a modified emulsifying method (Tanaka et al., 1963).

Preparation of NS. To 3.0 ml of sesame oil containing SO-15 (6.6 v/v%) and HCO-60 (1.5 v/v%), 0.3 ml of gelatin solution (30 w/v%) was added and both phases were emulsified at 70–80°C by sonification (100 W, 10 min). The drug (MMC or MMCD) or [^{131}I]-HSA was dissolved in gelatin solution before dispersing it into oil phase. The obtained emulsions were cooled in an ice bath to induce complete gelation of spheres, diluted with cold acetone, and then filtrated through polycarbonate membrane, 0.05 μm in pore size (Nuclepores, Calif., U.S.A.). For complete removal of adhering oil phase, NS was successively washed with acetone. The NS thus obtained were treated with 3.0 ml of formaldehyde dissolved in acetone (10%) for 10 min to harden the matrix followed by a washing with acetone and an air-drying. The final product was in the form of discrete spherical units and recovered as a free-flowing powder.

Preparation of MS. To 3.0 ml of corn oil, gelatin solution (0.3 ml, 30 w/v%) was added

and dispersed by vigorous shaking with vortex mixer, and then proceeded as described above. Polycarbonate membrane of 3 μm in pore size was used for filtration. The final product was also obtained as a free-flowing powder.

The content of MMC and MMCD in both spheres amounted 20 μg equivalent MMC/mg of NS or MS. Just before the test, both NS and MS were suspended with saline solution dissolving the dispersing agent (1% polysorbate 80) and then the mixture was ready for the injection.

In vitro release experiment

Five milligrams of NS or MS containing MMC in a free (NS–MMC, MS–MMC) or in conjugated form (NS–MMCD, MS–MMCD) was suspended in 5 ml of isotonic phosphate buffer (pH 7.4). The suspension was incubated at 37°C with slow shaking. At various periods of time, a sample was taken, filtered, and the amount of MMC released from the spheres was spectrophotometrically determined (ϵ = 22 000, λ_{max} = 364 nm).

Tissue distribution of [^{131}I]NS and [^{131}I]MS

Tissue distribution of [^{131}I]NS and [^{131}I]MS was determined according to the radioactivity distribution since [^{131}I]HSA was incorporated into the spheres as a tracer. Five milligrams of NS or MS containing 30 μg of [^{131}I]HSA was suspended in 1.0 ml of polysorbate saline solution and injected intravenously via the femoral vein to the rat under a light anesthesia with ether. At varying time periods after injection, rats were sacrificed and the organs such as the lung, liver, spleen, kidney, heart, thigh muscle and axial lymph node were excised. Blood samples were also withdrawn at the same time. The radioactivities of these tissues were determined by a well NaI-scintillation counter.

Tissue distribution of MMC or MMCD entrapped in NS or MS

Five milligrams of NS or MS containing MMC or MMCD was injected to the rat as described above. The dose of MMC was 100 μg per rat. After the injection, rats were sacrificed periodically and the organs were excised. The organs were weighed, homogenized in 10 ml of isotonic phosphate buffer solution (pH 8.0) and after centrifugation the supernatant was used for microbiological assay. The recovery of free MMC from the gelatin spheres was almost complete after these procedures.

The antimicrobial activity of each sample was assayed by the disc-plate method using *E. coli* B as a test organism. The concentration of MMC was calculated employing a standard curve in which MMC standard was added to an excised fresh organ followed by the same analytical procedure. In the case of MMCD-containing formulations, the tissue homogenate was divided into two samples. One sample was used for bioassay without any additional treatment and the other sample was boiled for 5 min to hydrolyze the MMCD to MMC before microbial analysis. The amount of free MMC and conjugated MMC were calculated from these values using the standard curve of tissue samples adding various amount of MMC and MMCD.

Histological examination

At various times after intravenous injection of MS, the lung was excised, fixed in 10% formaldehyde solution, stained with hematoxylin and eosin, and microscopically examined.

Antitumor activity analysis

For examining antitumor activity of MMC and MMCD incorporated in spheres, BDF_1 mice were inoculated intraperitoneally with a suspension of 1×10^6 P388 leukemia cells and the chemotherapy was given intraperitoneally 24 h after inoculation. All activities were calculated as T/C%, the ratio of the mean survival time of the treated group (T) divided by that of the control group (C).

RESULTS

Size estimation of NS and MS

Fig. 1A and B are an electron micrograph of NS and an optical photomicrograph of MS, respectively. The shapes of both types of spheres were invariably spherical. From these photographs, the sizes of about 500 spheres were estimated for either preparation and the size distribution was obtained as illustrated in Fig. 2. The diameter of NS distributed between 100 and 600 nm and the arithmetic average of particle diameter was 280 nm. The sizes of MS varied from 5 to 30 μm and the average diameter was of 14.9 μm. In the following experiments, batches having fairly similar average diameters of 230–350 nm (NS) and 12–19 μm (MS), were used.

In vitro release of MMC from NS and MS

Fig. 3 shows the release of MMC from NS–MMC and MS–MMC. Results are shown by a semilogarithmic plot of MMC remaining in the spheres as per cent of the initial amount at every sampling time. MMC was liberated from NS very rapidly and more than 50% of the initial amount of MMC was recovered in the incubation medium, 10 min after the incubation. On the contrary, gradual release of MMC was observed in the case of MS and it took about 2 h for releasing 50% of the incorporated MMC.

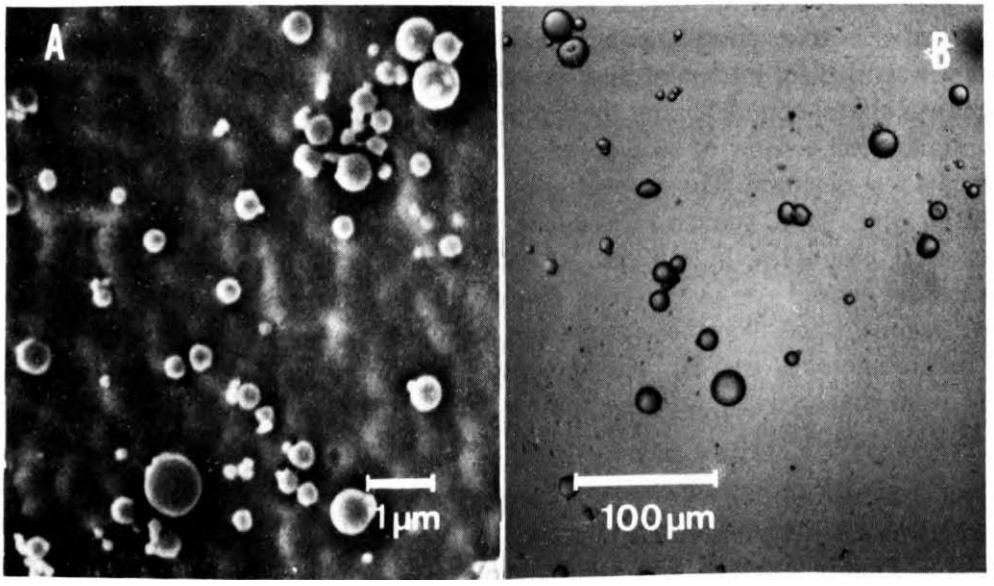

Fig. 1. Scanning electron micrograph of NS (A) and optical photomicrograph of MS (B).

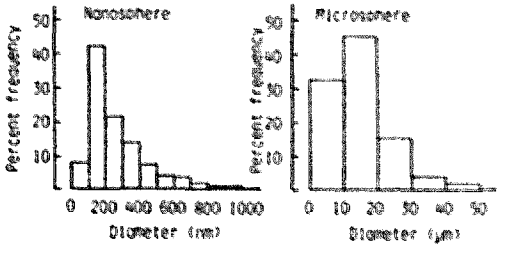

Fig. 2. Particle size distribution of NS and MS.

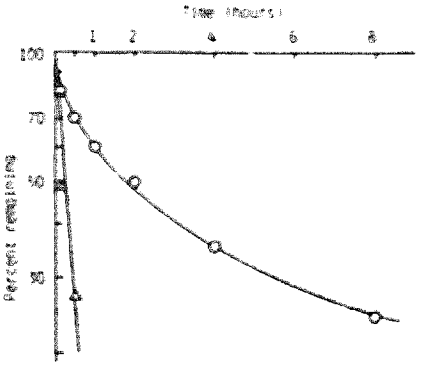

Fig. 3. In vitro release of MMS from NS–MMC and MS–MMC. △, NS; ○, MS. Results are expressed as the mean of 3 experiments.

The release of MMCD as the conjugated form from spheres was confirmed to be negligible. In contrast with the release patterns of MMC entrapped as the free form, NS and MS containing the dextran conjugate of MMC showed markedly slow release of MMC following monoexponential kinetics with a half-life of about 30 h. Each point represents the average of 3 experiments of one batch, but the coefficient of variance was less than 20% within batches tested.

Tissue distribution of [^{131}I]NS and [^{131}I]MS

The distribution of [^{131}I]NS and [^{131}I]MS was studied both quantitatively and histologically. The quantitative distribution in different organs was examined for 24 h after the intravenous administration of [^{131}I]-labeled spheres. The stability of radioactive label was ascertained by incubating [^{131}I]NS and [^{131}I]MS in phosphate buffer (pH 7.4) and plasma (20%) at 37°C. Since no significant liberation of [^{131}I]radioactivity was found within 24 h, the change of radioactivity in each organ could be regarded as the phenomenon exhibited by the accumulation and elimination of spheres themselves. Thus, the radioactivity biodistribution in the lung, liver, spleen, kidney, heart, and blood are summarized in Table 1. The values obtained from the thigh mucles and lymph nodes at every sampling were very low and therefore, not included in the table.

As is obvious from Table 1, [^{131}I]NS was taken up selectively by the reticuloendothelial system (RES) and 60% of the total dose was detected in the liver, 10 min after the administration. The radioactivity of NS in the liver decreased rapidly, and only 5.6% of the dose could be detected at 24 h following injection. On the other hand, more than 95% of the [^{131}I]MS selectively accumulated in the lung followed by a very slow elimination and 24 h after injection, approximately 60% of the dose still remained in the same organ.

Fig. 5A and B are photomicrographs of the lung 10 min and 2 weeks after intravenous injection of MS. Immediately after injection, MS had already reached the lung and lodged in precapillary arterioles and capillaries (Fig. 5A). The number of spheres obstructing

TABLE 1

TISSUE DISTRIBUTION OF [^{131}I]LABELED NS AND MS AT VARIOUS TIME PERIODS AFTER INTRAVENOUS INJECTION

	NS [a]			MS		
	10 min (5) [b]	2 h (5)	24 h (4)	10 min (4)	2 h (5)	24 h (4)
Lung	6.2 ± 1.1 [c]	3.5 ± 0.9	0.7 ± 0.2	95.4 ± 0.2	88.4 ± 22.0	59.9 ± 22.8
Liver	63.8 ± 1.1	44.2 ± 1.3	5.6 ± 1.2	3.6 ± 1.8	3.4 ± 0.7	3.6 ± 1.8
Kidney	0.7 ± 0.1	0.4 ± 0.0	0.3 ± 0.0	0.2 ± 0.2	0.3 ± 0.0	0.4 ± 0.2
Spleen	5.5 ± 2.2	6.0 ± 2.4	0.8 ± 0.2	0.2 ± 0.2	0.2 ± 0.0	0.5 ± 0.2
Heart	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.2	0.1 ± 0.0	ND [d]
Blood	0.3 ± 0.2	0.4 ± 0.2	0.4 ± 0.2	0.1 ± 0.0	0.1 ± 0.0	0.4 ± 0.4

[a] Total radioactivity in each organ is expressed as a percentage of dose except for blood. Blood concentration was expressed as a percentage of dose per one milliliter blood.
[b] Number of rats.
[c] Results are expressed as the mean ± S.D.
[d] Not detected.

capillaries decreased thereafter, and after 2 weeks no particle was observed on histological sections of the lung whereas inflammatory reactions could be still partially recognized (Fig. 5B). The considerably rapid metabolic degradation of gelatin spheres in vivo was indicated from these results.

Tissue distribution of MMC entrapped in NS and MS

Intravenous injection of free MMC in an aqueous solution or as NS–MMC showed similar biodistribution with no specific localization; the total amount of drug in those tissues was less than 1 μg (1% of the dose) at every period tested. In contrast, NS–MMCD exhibited selective accumulation of the drug in the liver reaching a level of 60 μg (60% of the injected dose) as shown in Fig. 6. At 2 h post-injection, however, no antimicrobial activ-

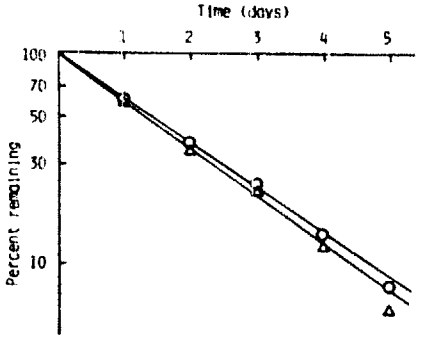

Fig. 4. In vitro release of MMC from NS–MMCD and MS–MMCD. △, NS; ○, MS. Results are expressed as the mean of 3 experiments.

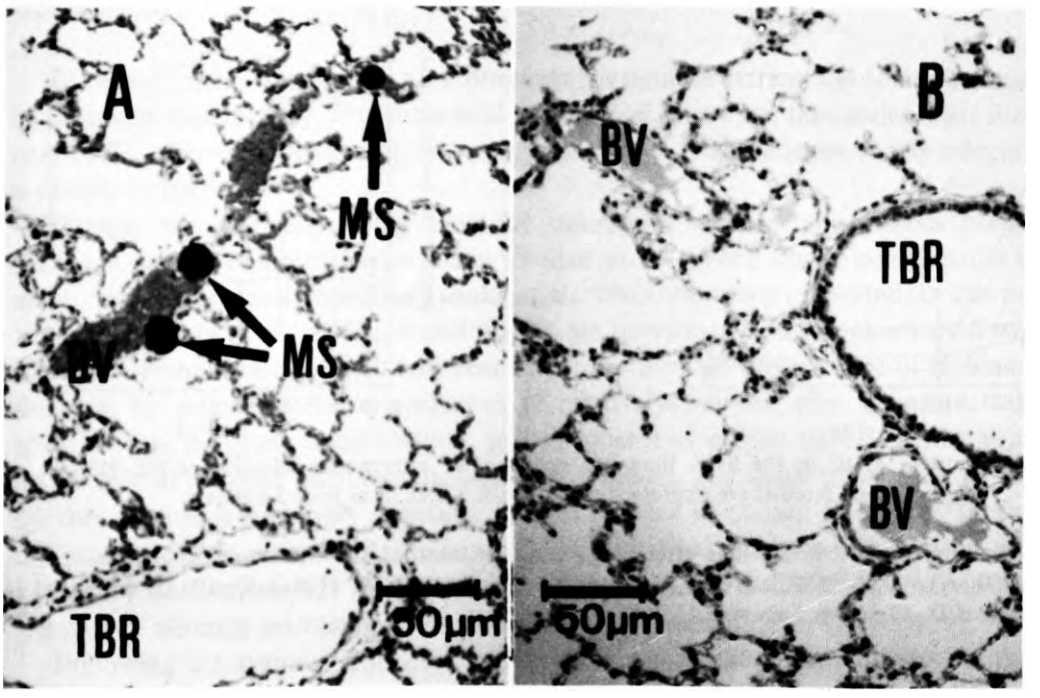

Fig. 5. Photomicrographs of the lung at 10 min (A) and 2 weeks (B) after intravenous injection of MS. TBR, terminal bronchioles, BV, blood vessels; MS, microspheres.

ity could be detected in the liver and only in the spleen and lung. Consequently, rapid inactivation of MMC in the liver was estimated.

The pulmonary distribution of MS–MMC and MS–MMCD are illustrated in Fig. 7. For comparison, the amount of MMC accumulated in the lung after the injection of 1 mg of free MMC in aqueous solution (a dose 10 times larger than the dose of the MS injection) is presented. After injection of MS–MMC, approximately 30 μg (30% of the dose) of MMC could be detected in the lung at both 10 min and 2 h. The amount of MMC detected in the lung decreased thereafter to less than 5 μg (5% of the dose) at 24 h after injection. On the contrary, more than 80 μg (80% of the dose) could be detected in the lung 10 min after the injection of MS–MMCD, and up to 60 μg (60% of the dose) of MMC was detected even at 24 h. Thus more gradual disappearance of MMC from the lung following injection of MS–MMCD was observed.

The antimicrobial activity of MMC in the plasma was also detected 10 min after the injection of MS–MMC but then it decreased rapidly and no antimicrobial activity could be detected after 2 h post-injection. However, the administration of MS–MMCD showed no antimicrobial activity in every plasma sample tested but in the lung. The injection of aqueous solution of MMC showed a considerably high plasma concentration but no antimicrobial activity in the organs.

Antitumor activity of MMC incorporated into NS and MS

Table 2 shows the effect of MMC administered with the new formulations on the survival time of BDF_1 mice bearing P388 leukemia. The average survival time of the untreated

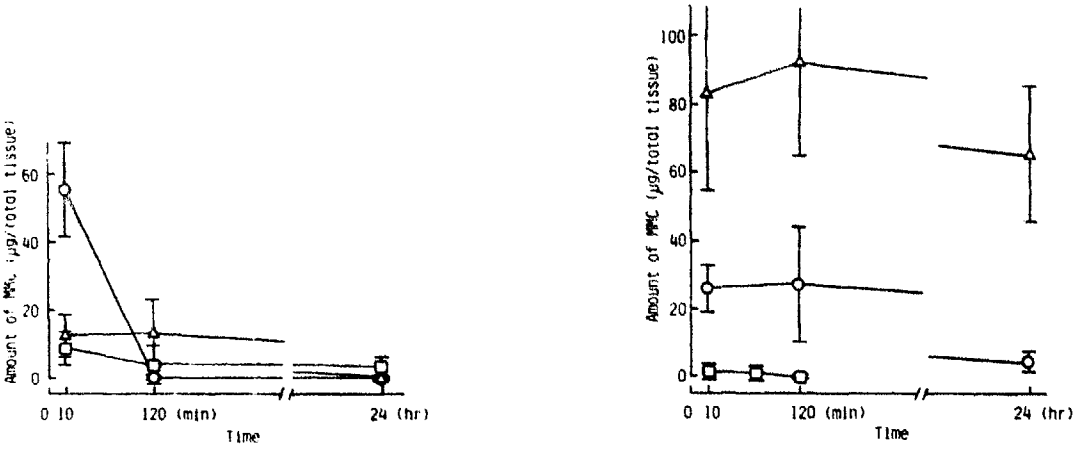

Fig. 6. Amount of MMC in the liver, lung and spleen after intravenous injection of NS–MMCD. ○, Liver; △, lung; □, spleen. Results are expressed as the mean ± S.D. of at least 5 animals.

Fig. 7. Amount of MMC in the lung after intravenous injection of MS–MMC or MS–MMCD. ○, MS–MMC (100 μg); △, MS–MMCD (100 μg); □, aqueous solution of MMC (1 mg). Results are expressed as the mean ± S.D. of at least 5 animals.

control group was 10.7 days. Treatment with NS–MMCD MS–MMC or MS–MMCD demonstrated an increase of the mean survival time and the ratio of the mean survival time of the treated to control group (T/C%) was 156%, 145% and 145%, respectively. These results are significantly larger than that of the control group ($P < 0.01$) but slightly smaller than those of aqueous solution injections ($P < 0.1$) so that the antitumor activity of MMC remained mostly inaltered even after incorporation into these preparations.

TABLE 2

EFFECT OF TREATMENT WITH VARIOUS FORMULATIONS OF MMC ON SURVIVAL TIME OF MICE BEARING P388 LEUKEMIA [a]

Formulation	Dose (mg equivalent MMC/kg)	Mean survival time ± S.D. (days)	T/C% [b]
Control [c] (10) [d]	0	10.7 ± 0.5	100
NS-MMCD (6)	10	16.7 ± 1.8	156
MS-MMC (6)	5	15.5 ± 1.9	145
MS-MMCD (6)	10	15.5 ± 1.6	145
MMC-Aqueous solution (6)	5	17.7 ± 1.2	165
MMCD-Aqueous solution (6)	10	18.3 ± 2.0	172

[a] P388 leukemia (1×10^6 cells) was inoculated intraperitoneally into BDF_1 mice. Chemotherapy was given by an intraperitoneal injection at 24 h after inoculation.
[b] The ratio of the mean survival time of the treated group (T) to that of the control group (C).
[c] Control group had no chemotherapy.
[d] Number of mice.

DISCUSSION

In recent years, encapsulation of drug into particulate carriers has found increased use in diagnosis and therapy. For successful application, however, the carrier itself should be non-toxic, biodegradable and of the appropriate shape and size to be widely adaptable for a variety of purposes.

Gelatin has been commonly used for microencapsulation and various preparation methods such as complex coacervation (Madan et al., 1974), simple coacervation (Nixon et al., 1968), and emulsification (Tanaka et al., 1963) have been reported. In our previous reports (Hashida et al., 1977a, b and 1979), we developed a microsphere-in-oil emulsion in which water-in-oil emulsion had been improved through replacement of its inner water droplets by gelled gelatin microspheres of relatively uniform size. By extracting these spheres from oil phase using acetone, gelatin spheres of various sizes, i.e. from about 100 nm to several hundred micrometers, could be obtained. The shape of the spheres prepared by this method is invariably spherical and the size can be chosen voluntarily by changing the emulsification procedure. The new process presented makes use of gelation of gelatin caused by cooling, so that the degradation of drugs could be minimized in contrast with the case of albumin microspheres in which heating is indispensable (Kramer, 1974).

Following intravenous injection, MS showed immediate accumulation in the lung, while NS was taken up predominantly by RES such as the liver and spleen. This correlation between particle size and distribution pattern was in good agreement with that of radiolabeled albumin microspheres which have been utilized for diagnostic scanning (Soloway and Davis, 1974). The uptake of MS by the lung can be explained by the trapping phenomenon at the pulmonary capillary bed before distributing to the whole body. NS, on the other hand, are phagocytized by RES after passing the pulmonary filter. Modification of relative organ distribution by controlling particle size was thus demonstrated. Concerning particle numbers in NS spheres, an amount of 1.5×10^5 times the number of MS particles can be estimated in a similar dose, but an assessment of the relation between particle number and in vivo disposition was difficult. However, since MMC could be delivered to the lung, liver and spleen according to the distribution patterns of NS and MS, it can be concluded that both spheres proficiently play the role of carrier.

From the results of in vitro release experiments, it became obvious that about 50% of MMC incorporated as the free form was liberated rapidly from NS, while the release from MS took about 2 h. On the contrary, MMC entrapped into the spheres as MMCD was slowly released and the rate was fairly similar to that of hydrolytic cleavage of MMC from dextran (Kojima et al., 1980), suggesting that the liberation process of MMC from dextran is a rate-limiting process in both spheres.

Thus the use of the present integrated procedure, i.e. the two step fixation method demonstrated the possibility of decreasing the release rate of the drug. In this approach, dextran, the backbone of the conjugate, played the role of an anchor which could moor MMC to the spherical carriers. The experiment carried out in vivo also revealed that MMC was gradually released from dextran fixed to spheres since an enhanced accumulation and a subsequent slow disappearance of MMC from the lung followed the injection of MS–MMCD (Fig. 7).

MMC has been one of the most extensively used anticancer agents but its systemic

treatment has only been reported to be of palliative benefit since it has always been necessary to halt therapy short of a cancer-sterilizing dose due to the toxicity. So, a specific carrier system which can deliver the drug to a desired target site is most desirable.

Based on the evidence presented in this investigation, the application of NS and MS as a drug delivery system of MMC seems advantageous in the treatment of tumors localized in the liver, spleen and lung due to the sustained release to those cumulative organs. Reduction of MMC concentration in plasma and in non-target organs is the most desirable feature to reduce adverse effects. In addition, gelatin spheres are biodegradable, less antigenic, and capable of incorporating a wide variety of drug molecules in a basically non-specific way, as well as being regarded as excellent delivery systems for cancer chemotherapy.

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